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Novel nanosized water soluble fluorescent micelles with embedded perylene diimide fluorophores for potential biomedical applications: Cell permeability, localization and cytotoxicity

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ABSTRACT

Novel biocompatible water-soluble fluorescent micelles with embedded perylene diimides (PDI) for intracellular applications have been prepared by self assembling of amphiphilic poly(vinyl alcohol)-*b*-poly(acrylonitrile) (PVA-*b*-PAN) copolymers in the presence of synthesized fluorophores. Amphiphilic PVA-*b*-PAN copolymers were obtained by selective hydrolysis of well-defined poly(vinyl acetate)-*b*-poly(acrylonitrile) (PVAc-*b*-PAN) copolymer. The preparation of the novel fluorescence micelles consisting of PVA hydrophilic shell and PAN hydrophobic core with incorporated PDI fluorophores has been confirmed by DLS and TEM analysis. The cytotoxicity of the water-soluble fluorophores and their internalization into living cells depending on the micellar concentration have been tested. It was shown that they could successfully enter in living cells without destroying their morphology. The results obtained indicate that the novel water-soluble fluorescent micelles with embedded PDI fluorophores would be suitable for potential intracellular biomedical applications.

1. Introduction

Perylene-3,4,9,10-tetracarboxylic diimides (PDI) have attracted an increasing interest during the past decades, and this can be traced back to their appealing properties, e.g., high electron affinity, large electron mobility, excellent thermal and oxidative stability, high molar absorptivity and quantum yield of fluorescence [1]. They are easy of synthetic modification [2] and widely used *n*-type materials for organic electronic devices such as solar cells [3–5], field-effect transistors [6–8], light-emitting diodes [9–11]. They have been also used in liquid crystals [12,13], as chemosensing materials [14–16] and dyes in photodynamic therapy [17]. Due to their unique properties PDI chromophores could be successfully applied in biological environment as high-performance fluorescent labels. The serious drawback of most of the synthetic dyes for their processing and material science applications is their low solubility in the common solvents and aqueous media [18,19]. Usually the solubility can be improved with the long-tail or swallow-tail conformation that is obtained by long alkyl substitutions in *N*-position of the dye [20–22]. For a biological application, the water solubility of PDI chromophores is essential. Different water-soluble PDI chromophores possessing hydrophilizing substituent as a sulfonic acid part [23], quaternized amine groups [24], polyethylene glycol attached to the chromophore [25] have been reported. In many cases, no fluorescence in water for these perylene chromophores has been detected. The inserting of polar side chains on the perylene bay-area is another way to enhance the water solubility of the PDIs and to reduce the amount of their self-aggregation [26,27] followed by encapsulation within dendritic shell thus preventing the possible intermolecular interaction [26]. Therefore, the choice of an appropriate synthetic strategy where the fluorescence properties of water soluble PDI chromophores will be retained and the self-aggregation will be reduced is of crucial importance. However, the synthetic approaches for preparation of such water soluble chromophores are often hard and multistep time consuming processes.

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One approach to overcome the problem with the low water solubility of PDI chromophores is to incorporate them into polymeric assemblies of nanoscale dimensions thus ensuring their water dispersibility and improved biocompatibility. The amphiphilic block copolymers which possess one hydrophilic and one hydrophobic block are capable of self-assembling in an aqueous solution thus forming different nanosized morphologies as micelles, nanospheres, and nanocapsules that can be used as nanoreactors to incorporate different compounds [28–31].

Most of the organic dyes work in the presence of organic solvents. The latter are environmental unfriendly and significantly restrict the dyes' practical application in living organisms. Therefore, the aim of the present study was the preparation of novel biocompatible water-soluble fluorescent micelles with embedded PDI fluorophores for potential intracellular applications.

2. Materials and methods

2.1. Materials

Perylene-3,4,9,10-tetracarboxy dianhydride, *N,N*-dimethylethylenediamine, 2,2,6,6-tetramethylpiperidin-4-ylamine and *n*-butylamine, (Aldrich), p.a. grade, were used without purification. All solvents (Aldrich, Fisher Chemical) were pure or of spectroscopy grade.

2.2. Analysis

FT-IR spectra were recorded on a Varian Scimitar 1000 spectrometer. The UV–VIS absorption spectra were recorded on a spectrophotometer Hewlett Packard 8452A. The corrected fluorescence spectra were taken on a Scinco FS-2 spectrofluorimeter at room temperature (25 °C). The NMR spectra were recorded on a Bruker DRX-250 spectrometer, operating at 250.13 MHz and 62.90 MHz for ^{1}H and ^{13}C, respectively, using a dual 5 mm probe head. The measurements were carried out in DMSO-d_6 solution at ambient temperature.

Transmission electron microscopy (TEM) observations were carried out with a JEOL instrument operating at a voltage of 100 kV. Samples were prepared by deposition of a droplet of the aqueous micellar solution onto a carbon coated copper TEM grid, which was allowed to evaporate for 2 h.

Dynamic light scattering (DLS) measurements were performed on a Brookhaven Instruments Corp. equipped with a He–Ne laser. The temperature was set to 22 °C and the angle of measurements was 90°. The measurements of the hydrodynamic diameter and particle size distribution as well as all other experiments were performed at room temperature after filtration of the aqueous micellar solution through a 0.45 μm filter.

2.3. Synthesis of PVA-b-PAN block copolymer by selective hydrolysis of PVAc-b-PAN.

$PVAc_{269}$-*b*-PAN_{231} block copolymer was prepared following the procedure described in reference [32]. In brief, poly(vinyl acetate) $_{269}$-*b*-poly(acrilonitrile) $_{231}$ ($PVAc_{269}$-*b*-PAN_{231}) block copolymer (1.0 g) was dissolved in DMSO (5 mL) and added into a solution of potassium hydroxide (4.5 g) in methanol (50 mL, p.a.). The molar amount of KOH relative to the VAc units was 9:1. After stirring for 48 h at room temperature, the PVA-*b*-PAN copolymer was collected, washed with ethyl acetate and dried in vacuo at 50 °C. ^{1}H NMR (DMSO-d_6, 250.13 MHz) ppm: 4.15–4.70 (1H, CH_2-CH-O*H* of PVA); 3.85 (1H, CH_2–C*H*–OH of PVA); 3.18 (1H, CH_2–C*H*–CN of PAN); 1.75–2.20 (2H, C*H*$_2$–CH–CN of PAN); 1.10–1.62 ppm (2H, C*H*$_2$–CH–OH of PVA). ^{13}C NMR (DMSO-d_6, 62.90 MHz) ppm: 124.2 (CH_2–CH–*C*N of PAN); 68.1–72.3 (CH_2–*C*H–OH of PVA); 51.4 (*C*H$_2$–CH–OH of PVA); 37.6 (CH_2–*C*H–CN of PAN); 32.1 (*C*H$_2$–CH–CN of PAN).

The level of hydrolysis (ca. 98%) of the poly(vinyl alcohol) block was calculated using ^{1}H NMR by comparing the ratio of the peaks areas for the PVAc (CH_2–C*H*–$OCOCH_3$) at 4.92 ppm and PAN (CH_2–C*H*–CN) at 3.18 ppm units in the original copolymers and those for the PVA (CH_2–C*H*–OH) at 3.85 ppm and PAN (CH_2–C*H*–CN) at 3.18 ppm units in the final PVA-*b*-PAN copolymers.

2.4. Synthesis of perylene-3,4,9,10-tetracarboxylic diimides (**2–4**)

To a suspension of 2.0 g (5 mmol) perylene-3,4,9,10-tetracarboxylic dianhydride **1** into a mixture of 50 mL of water and 50 mL of *n*-propanol, 20 mmol of the corresponding primary amine (2.1 mL of *N,N*-dimethylethylenediamine, 2.0 mL of *n*-butylamine, or 3.5 mL of 2,2,6,6-tetramethylpiperidin-4-ylamine) was added. The resulting mixture was heated under reflux for 4 h. After cooling to room temperature the precipitate was collected by filtration, washed with water and dried. Then the crude solid was treated with 100 mL of 5% aqueous sodium hydroxide, washed with warm water and filtered off. The pure compound was further dried at room temperature.

PDI 2: Yield — 2.42 g (91%). FT-IR (KBr) cm^{-1}: 2962 and 2770 (νCH); 1694 (ν^{as}N–C═O); 1658 (ν^{s}N–C═O). Elemental analysis: Calculated for $C_{32}H_{28}N_4O_4$ (MW 532.59) C 72.16, H 5.30, N 10.52%; found C 71.89, H 5.22, N 10.85%.

PDI 3: Yield — 2.36 g (94%). FT-IR (KBr) cm^{-1}: 2948 (νCH); 1696 (ν^{as}N–C═O); 1659 (ν^{s}N–C═O). Elemental analysis: Calculated for $C_{32}H_{26}N_2O_4$ (MW 502,56) C 76.48, H 5.21, N 5.57%; found C 76.24, H 5.07, N 5.77%.

PDI 4: FT-IR (KBr) cm^{-1}: Yield — 2.78 g (83%). 3385 (νNH); 2961 (νCH); 1686 (ν^{as}N–C═O); 1650 (ν^{s}N–C═O). Elemental analysis: Calculated for $C_{42}H_{44}N_4O_4$ (MW 668,82) C 75.42, H 6.63, N 8.38%; Found C 75.19, H 6.46, N 8.59%.

2.5. Synthesis of PDI/PVA-b-PAN micelles

To 2 mL of a PVA_{269}-*b*-PAN_{231} copolymer solution in DMSO (c = 10 mg mL^{-1}), and 0.002 g (**sample 1**) or 0.02 g (**sample 2**) PDI (**2–4**) dissolved in 5 mL of DMF were added. The solution was stirred for 24 h then slowly added to 13 mL of deionized water at a flow rate of ca. 8 mL h^{-1} and stirred for additional 24 h. Dialysis against deionized water for 48 h was performed in order to eliminate DMSO and non-incorporated fluorophores.

2.6. MTT test for cell survival

To analyze the cytotoxic effect of PDI/PVA_{269}-*b*-PAN_{231} micelles on the 3T3 fibroblasts cells, the MTT test (Invitrogen, USA) was performed as described by Mosmann [33], with some modifications. The adherent cells were treated with 0.05 mg/mL, 0.15 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 0.75 mg/mL solutions of PDI/PVA_{269}-*b*-PAN_{231} micelles and incubated additionally for 24 or 48 h. As a control, cells non-treated with PDI/PVA_{269}-*b*-PAN_{231} micelles were used. After the incubation period the cell medium was changed with fresh medium (200 μL/well). Then, 50 μL of MTT solution (5 mg/mL in PBS) was added. Plates were further incubated for 4 h at 37 °C and the formed formazan crystals were dissolved by addition of 250 μL solvent (5% formic acid in 2-propanol) per well and mixing. The absorbance was recorded at 570 nm with the 96-well plate reader Tecan Infinite F200 PRO (Tecan Austria GmbH, Salzburg). For each concentration six wells were used. Complete medium (200 μL) and 5% formic acid in 2-propanol (250 μL) were used as a blank solution.

2.7. Cell treatment with PDI/PVA-b-PAN micelles and fluorescent imaging

After 24 h of incubation the cells were treated with 0.05 mg/mL, 0.15 mg/mL, 0.25 mg/mL and 0.5 mg/mL solutions of PDI/PVA_{269}-*b*-PAN_{231} micelles and were incubated additionally for 4 or 24 h. The